Eur. J. Biochem. 238, 476–483 (1996)

Developmental expression of *CYP2E1* in the human liver
Hypermethylation control of gene expression during the neonatal period

Isabel VIEIRA, Michelle SONNIER and Thierry CRESTEIL

INSERM U75, Université René Descartes, Paris, France

(Received 8 January/11 March 1996) – EJB 96 0024/1

Cytochromes *P*-450 are responsible for the biotransformation of drugs and other hydrophobic molecules by the liver. Several isoforms coexist which display an asynchronous onset during the perinatal period suggesting the involvement of multiple mechanisms of regulation. In this paper, we have shown that the CYP2E1 protein and its associated activity could not be detected in the fetal liver and rise during the first few hours following birth independently of the gestational age (between 25–40 weeks). During this period, the CYP2E1 RNA content remains fairly low: the stabilization of the low amount of existing CYP2E1 protein by endogenous ketone bodies could explain the early neonatal rise of the protein level. From 1 month to 1 year, the protein content gradually increases and is accompanied by the accumulation of CYP2E1 RNA, suggesting a transcriptional activation of the gene during the late neonatal period.

We examined the methylation status of CpG residues in the 5′ flanking region, first exon and first intron of *CYP2E1* gene cleaved with *Hpa*II/*Msp*I. Genomic DNA from fetal liver shows several hypermethylated spots in the first-exon–first-intron region, which progressively disappear in neonatal samples. We conclude that during the neonatal period, the accumulation of hepatic CYP2E1 RNA is correlated with the degree of methylation at the 5′ end of the *CYP2E1* gene.

Keywords: human liver; *CYP2E1*; hypermethylation; neonatal period; regulation.

The cytochrome *P*-450 (*P*-450) gene superfamily (CYP) encodes a group of hemoproteins that mostly catalyze the oxidative metabolism (or monooxygenation) of hydrophobic endogenous compounds like steroids, fatty acids, prostaglandins and exogenous chemicals including drugs, carcinogens and environmental pollutants [1]. These substrates can be converted either to inert polar metabolites further eliminated in a water-soluble form or to cytotoxic or carcinogenic derivatives [2]. *CYP2E1* is a member of this superfamily mainly expressed in the human liver and to a minor extent in the lung [3]; traces of CYP2E1 have also been detected in the small intestine [4] and in the brain [5]. CYP2E1 catalyzes the metabolic activation of chemicals presenting a wide structural diversity, including aromatic compounds, benzene, *N*-nitrosodimethylamine, halogenated alkanes and others low-molecular-mass compounds which have toxicological implications in humans [6–8]. It has also been implicated in the metabolism of drugs and halogenated anesthetics, like acetaminophen, isoniazid, halothane and enflurane [9, 10]. Additionally, CYP2E1 is involved in the metabolism of alcohols, aldehydes, ketones and plays a key role in gluconeogenesis from endogenous ketone bodies released in situations involving hormonal or metabolic changes associated with energy deprivation [11–13].

A general feature of the monooxygenase system is its ability to be activated by exogenous compounds. In this respect, the CYP2E1 content is increased by a set of chemicals, including its own substrates acetone, ethanol, isoniazid or pyrazole, but also by fasting and diabetes [14–17]. This could have a considerable importance for the synergistic action of alcohol consumption on the activation and toxicity of drugs and procarcinogens [15]. In rat, CYP2E1 expression has been demonstrated to be controlled at three levels: post-translational by a ligand-dependent enzyme stabilization, translational by stabilization of its RNA and transcriptional during ontogenesis [18–21]. The CYP2E1 RNA surges immediately after birth in relation with the activation of gene transcription and is associated with specific demethylation of CpG residues in the 5′ flanking region [21–23].

CYP2E1 is a major *P*-450 isoform expressed in the human liver [6, 24]. The human gene is present as a single copy on chromosome 10 (10q24.3-qter). Its complete sequence and the structure of its promoter have been documented by Umeno et al. [25]. It is composed of nine exons and spans 11413 bp. A canonical TATA box and a CCATT box have been located 23 bp and 144 bp upstream the transcription start site.

In the present study, we investigated the expression of *CYP2E1* during human liver development. Our results showed that the CYP2E1 protein was absent from the fetal liver and rose immediately after birth, regardless of the gestational age. The immunochemical level of the protein and its catalytic activity steadily increased during the first year. We also observed that during ontogenesis, CYP2E1 RNA levels remained low during the early postnatal period, the major accumulation of the transcript occurring between 1–3 months of age. To determine whether the developmental pattern of *CYP2E1* expression is correlated with a specific demethylation of CpG residues, we analyzed the restriction profile obtained by cleavage of genomic DNA with methylation-sensitive endonucleases *Hpa*II and *Msp*I.

Correspondence to T. Cresteil, INSERM U75, 156 rue de Vaugirard, F-75730 Paris cedex 15, France

Fax: +33 1 45 67 46 72.

Abbreviations. NaCl/Cit, 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0; NaCl/P_i/EDTA, 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA, pH 7.4; *P*-450 or CYP, cytochrome *P*-450.

Enzymes. Cytochrome *P*-450 (EC 1.14.14.1); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

Our results suggest transcriptional activation of the human *CYP2E1* gene through the demethylation of CpG residues within the first exon and first intron.

METHODS

Tissue collection. Liver samples were obtained postmortem, within the first hours after death, from fetuses, newborns, infants and adults, under the supervision of the Ethical Committee from *Institut National de la Santé et de la Recherche Médicale* (INSERM). Adult liver samples were obtained from donors for kidney transplantation. Donors had no severe chronic pathology and had generally died from a traffic accident. They had no repeated drug consumption. No information was available regarding their smoking and drinking habits. Fetuses either stillborn or aborted for medical reasons (Down's syndrome, hydramnios, severe hypotrophy, malformations) were aged 16–40 weeks. Liver samples were collected from children aged less than one day to 9 years with informed parental consent. These children suffered from severe infection, respiratory distress resulting from immature pulmonary function, hypertension, hydro- or micro-cephaly, non-reversible hypotrophy or sudden death. After excision, samples were immediately frozen in liquid nitrogen and stored at −80 °C until further processing.

To facilitate the analysis of results, samples were grouped according to their gestational or postnatal age: fetuses aged less than 30 weeks of gestational age (n = 66, 23.2 ± 3.6 weeks); fetuses aged more than 30 weeks (n = 16, 35.3 ± 2.8 weeks); newborns aged less than 24 h (n = 13, gestational age at birth 30.0 ± 3.5 weeks, postnatal age 0.5 ± 0.25 day); newborns aged 1–7 days (n = 20, gestational age at birth 32.4 ± 4.5 weeks, postnatal age 3.9 ± 2.0 days); newborns aged 8–28 days (n = 21, gestational age at birth 32.4 ± 5.4 weeks, postnatal age 17.9 ± 12.2 days); newborns aged 1–3 months (n = 26, gestational age at birth 34.2 ± 6.0 weeks, postnatal age 60.5 ± 23 days); newborns aged 3–12 months (n = 18, 166 ± 67 days); infants aged 1–10 years (n = 7, 2273 ± 1203 days) and finally adult samples (n = 16).

Preparation of microsomal fractions and determination of CYP2E1 content. Frozen liver samples were thawed in ice-cold saline and homogenized at 4 °C in 100 mM Tris/HCl pH 7.4, 250 mM sucrose, 5 mM EDTA. Microsomal fractions were prepared by differential centrifugation and resuspended in 100 mM sodium phosphate pH 7.4 containing 20% glycerol and stored at −80 °C [26]. Protein concentration was measured by the procedure of Lowry et al. [27]; the total *P*-450 content was estimated according to Greim [28]. Preparations showing evidence of protein degradation were discarded from the study as reported elsewhere [29].

Microsomal proteins (60 µg) were separated on 9% SDS/PAGE [30] and transferred onto a nitrocellulose Hybond C membrane (Amersham) overnight at 0.8 mA/cm² to achieve complete transfer [31]. CYP2E1 protein was detected immunochemically with a polyclonal antibody raised against the rat orthologue CYP2E (Oxygene, Dallas TX) diluted 1/200. The antigen-antibody complex was visualized after addition of peroxidase-conjugated anti-(rabbit IgG) antibodies diluted 1/500, using 4-chloro-1-naphthol as dye. A single band was detected under these conditions. The amount of reacting material was quantified by scanning the membrane with a computerized image-analyzing system (IMSTAR, France). Reference adult samples were routinely incorporated in each experiment to calibrate determinations. Results were expressed as absorbance/mg microsomal protein.

Chlorzoxazone hydroxylase activity. For the chlorzoxazone hydroxylase assay, 450–600 µg microsomal protein was incubated for 3 min at 37 °C in 1 ml 0.1 M potassium phosphate pH 7.2, containing 500 µM of chlorzoxazone and a NADPH-generating system (13.7 mM glucose 6-phosphate, 0.66 mM NADP). In our conditions, the reaction was linear up to 40 min with adult microsomal preparations. The reaction was initiated by addition of glucose 6-phosphate dehydrogenase (2.8 IU/ml), incubated for 30 min at 37 °C and stopped by 50 µl 43% (by vol.) H_3PO_4. After extraction with dichloromethane and evaporation to dryness under nitrogen, residues were dissolved in 100 µl CH_3CN/H_2O (4:6, by vol.) and 20-µl aliquots were analyzed by HPLC using a C8 reverse-phase column, according to the procedure of Peter et al. [32]. Elution was realized at a flow rate of 1.8 ml/min by a mixture of CH_3CN/0.5% H_3PO_4 (25:75, by vol.). Retention times of chlorzoxazone and its 6-hydroxylated metabolite, monitored at 287 nm, were 8.2 min and 2.4 min, respectively. Chlorzoxazone hydroxylase inhibition was studied with 100, 250 and 500 µM diethyldithiocarbamate added to the incubation mixture.

RNA isolation and slot-blot analysis. Total RNA was isolated from 50–100 mg human liver samples, following the procedure described by Chomczynsky et al. [33]. The presence of 28S and 18S ribosomal RNA as distinct bands with a very limited smear in a denaturing agarose gel allowed the integrity of RNA preparations to be assessed. For slot-blot analysis, 10 µg total RNA was applied to Hybond N membrane (Amersham). The blots were prehybridized for 6 h at 65 °C in 6×NaCl/Cit (1×NaCl = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 0.5% SDS, 5×Denhardt's solution and 100 µg/ml denatured salmon sperm DNA. The hybridization was performed overnight at 65 °C, in the same solution containing 10^6 cpm/ml of the labeled probe B2 (see below). Membranes were washed under stringent conditions in 0.1×NaCl/Cit, 0.1% SDS for 15 min at 65 °C and exposed overnight to Kodak XAR 5 films at −80 °C in the presence of intensifying screens. The blots were stripped and reprobed with probe C in the same conditions. Finally, after stripping, all blots were hybridized with a 26-bp oligonucleotide probe specific for the human 28S rRNA [34], 5′-end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase [35]. Quantification of the CYP2E1 transcripts was achieved by scanning of autoradiograms with an image analysis system (IMSTAR) and normalized to the 28S rRNA content.

Probes. The probes used in this study were prepared by the polymerase chain reaction (PCR). Genomic DNA extracted from human liver was used as template for amplification of three fragments corresponding to the 5′ flanking region (fragment A, 1.1 kbp), to exons 1–2 (fragment B, 1.7 kbp) and to exon 5 (fragment C, 170 bp) of the *CYP2E1* gene. The primers were designed according to published nucleotide sequences [21, 25, 36]. PCR was performed under the following conditions: 250 ng genomic DNA was amplified in 50 µl 50 mM KCl, 10 mM Tris/HCl pH 9.0, 0.1% Triton X-100, 2 mM $MgCl_2$, containing 500 µM each of four dNTPs, and 25 pmol downstream and upstream primers. After denaturation at 95 °C for 10 min, the reaction was initiated by the addition of 1.25 U *Taq* DNA polymerase (Promega). Amplification was run for 40 cycles followed by a further elongation at 72 °C for 15 min with the following optimized amplification conditions.

Fragment A. Denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C for 2 min. Primers for amplification were: 5′-TGCAACCTATGAATTAAGAAC-3′ (sense strand, positions −1013 to −993 [36]) and 5′-CACCAG-CAGGGCCACGGTGAC-3′ (antisense strand, positions 52–72).

Fragment B. Denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 3 min. Primers were designed from the human *CYP2E1* sequence [25]: 5′-GTCCAA-

CTCCATCCTCACCAG-3′ (sense strand, positions −197 to −177) and 5′-CCATAAAACTCTCCCGCACAG-3′ (antisense strand, positions 1493−1513).

Fragment C. Amplification was run under the conditions described for fragment A, except the primer annealing at 52 °C for 1 min. Primers were 5′-TTCCCAGCTTTCTACACTACTT-3′ (sense strand, positions 5338−5359) and 5′-GCCTACCTTCTC-CATTTCCA-3′ (antisense strand, positions 5488−5507).

The fragment A was directly subcloned in pCR™ (InVitrogen, SanDiego CA) and sequenced by the dideoxynucleotide chain-termination method using T7 polymerase (Pharmacia). The fragment B was separately cleaved by *Xba*I, *Sfi*I and *Fsp*I/*Xba*I; only one band was purified from each digestion: fragments of 1.2 kbp (B1), 600 bp (B2) and 500 bp (B3). The probes were labeled with [α-^{32}P]dCTP to a specific activity of 0.5−2×10^9 cpm/μg with the random primer kit from Amersham.

Genomic DNA isolation and methylation analysis. Genomic DNA was isolated from human liver samples according to the method described previously [35]. The quality of DNA preparations was checked by estimation of the average molecular mass of DNA after migration on 0.3% agarose gel electrophoresis. The purity of DNA was estimated by spectrophotometry and only preparations with a ratio $A_{260}/A_{280} > 1.8$ were kept for analysis.

To examine the degree of methylation, 30 μg genomic DNA was digested with *Hpa*II or *Msp*I under the conditions recommended by manufacturer (MBI Fermentas, Lithuania), using a 3−5-fold enzyme excess. Restriction enzymes were added in two aliquots, corresponding to two-thirds and one-third of the total enzyme amount, with a delay of 2−5 h to ensure that digestion was run to completion. Digestion products were salt/ethanol-precipitated and separated by electrophoresis on a 1.8% agarose gel followed by transfer to Hybond N membranes. The membranes were prehybridized for 6 h at 65 °C in 5×NaCl/P$_i$/EDTA (1×NaCl/P$_i$/EDTA = 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA, pH 7.4), 0.1% SDS, 5×Denhardt's solution, 100 μg/ml denatured salmon sperm DNA and hybridized overnight in the same solution containing 10^6 cpm/ml of the radiolabeled probes. The filters were washed twice for 10 min in 1×NaCl/P$_i$/EDTA, 0.1% SDS at 65 °C and then 15 min at 65 °C in 0.5×NaCl/P$_i$/EDTA, 0.1% SDS. Dried membranes were exposed to X-ray films with intensifying screens for 5 days.

RESULTS

CYP2E1 protein ontogenesis. In order to explore the ontogenesis of CYP2E1, we first determined its protein level in 107 hepatic samples obtained at different stages of development. Using a polyclonal antibody raised against the rat orthologue CYP2E1, we observed a single band in immunoblots. We did not detect the CYP2E1 protein in liver microsomes from fetuses aged 14−40 weeks of gestational age (e.g. <0.2 density unit · mg^{-1} protein), whatever the causes of abortion (spontaneous or therapeutic). The microsomal content in CYP2E1 rose immediately after birth, during the first day of extra-uterine life, independently of the gestational age at birth ranged over 25−40 weeks (Fig. 1). The protein level gradually increased during the first year to reach the adult value in infants aged 1−10 years. This developmental pattern of *CYP2E1* expression was not correlated with the evolution profile of the spectrophotometrically determined total cytochrome *P*-450 content: it remained fairly stable from the 17th week of gestation to one year of age at approximately 40% of the adult level (data not shown).

Chlorzoxazone metabolism. For monitoring the level of catalytically active enzyme, we examined the *in vitro* hydroxylation

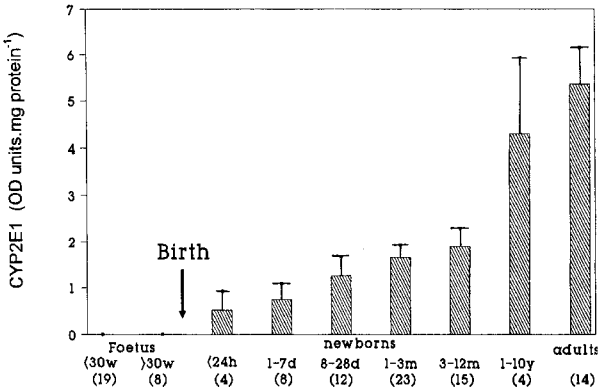

Fig. 1. Age-related variations of CYP2E1 protein in the human liver. Microsomal proteins (60 μg) were separated on a 9% SDS/PAGE and transferred to nylon membranes. CYP2E1 was immunochemically detected with a polyclonal antibody raised against rat orthologue CYP2E1; the antigen-antibody complex was visualized after addition of a peroxidase-conjugated anti-(rabbit IgG) antibody using 4-chloro-1-naphthol as the dye, and quantified by scanning with an image analysis system. Results are expressed as the mean ± SE of densitometric values of immunochemically detected CYP2E1/mg protein; OD units are an arbitrary measure of the density. Groups are defined in Methods and the number of samples in each group is indicated between brackets.

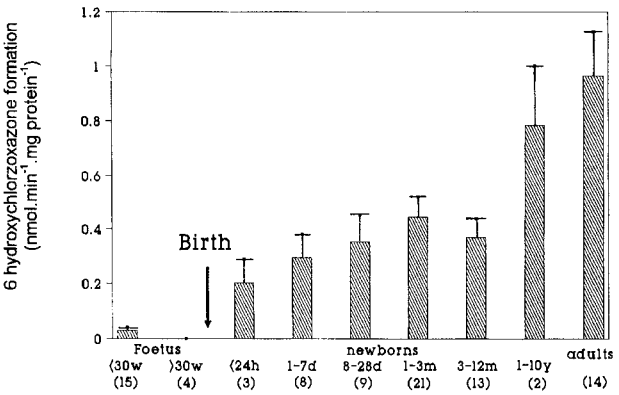

Fig. 2. Age-related variations of chlorzoxazone hydroxylation in the human liver. Microsomal samples (0.3 nmol *P*-450) were incubated with 500 μM chlorzoxazone and a NADPH-generating system. The formation of the 6-hydroxylated metabolite was monitored at 287 nm after separation by HPLC. Results are expressed as the mean ± SE of activity measured as rate of formation of 6-hydroxychlorzoxazone/mg microsomal protein. Groups are defined in Methods and the number of samples in each group is indicated between brackets.

of chlorzoxazone, a relatively specific probe for human CYP2E1 in 89 microsomal preparations. Only CYP1A1 could interfere in the assay [37], but it is generally assumed that CYP1A1 is not expressed in appreciable amounts in human livers. Results are expressed as rate of formation of 6-hydroxychlorzoxazone/mg microsomal proteins. In fetal microsomes, the absence of immunodetectable CYP2E1 was associated with the extremely low level of enzyme activity (Fig. 2). The chlorzoxazone hydroxylase activity rose during the first 24 h following birth and steadily increased during the first year. Activity reached adult value in infants aged 1−10 years. This profile of evolution paralleled the increase in CYP2E1 protein during development: therefore, a close relationship was observed ($r = 0.778$; $P < 0.001$) between immunochemical determination of CYP2E1 and chlorzoxazone hydroxylase activity.

To further investigate the properties of CYP2E1, the kinetic parameters K_m and V_{max} of chlorzoxazone hydroxylase activity

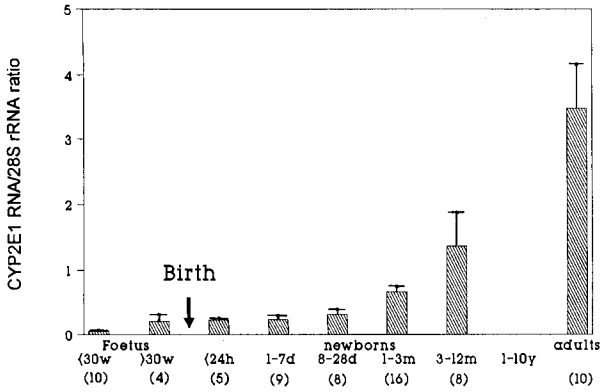

Fig. 3. Age-related variations of CYP2E1 RNA in the human liver. Total RNA was extracted from human livers and 10 μg was applied to nylon membranes for slot-blot hybridization. Blots were hybridized overnight at 65 °C with ^{32}P-labeled probe C corresponding to exon 5. Membranes were washed under stringent conditions (0.1×NaCl/Cit, 0.1% SDS at 65 °C) and exposed to Kodak XAR5 films. Membranes were stripped and probed again with a 26-bp oligonucleotide for human 28S rRNA. After scanning of autoradiograms, results are expressed as the mean ± SE of the ratio of densitometric values of CYP2E1 RNA normalized to 28S rRNA. Groups are defined in Methods and the number of samples in each group is indicated between brackets.

were measured in both neonatal and adult microsomal preparations. The K_m values obtained were 28.8 μM in newborns and 15.8 μM in adults; the V_{max} values were 0.96 and 1.0 nmol · mg^{-1} · min^{-1}, respectively. These values were comparable in all samples studied which means that the CYP2E1 protein exhibited the same catalytic properties in newborn and adult livers.

Finally, we studied the *in vitro* inhibition of chlorzoxazone hydroxylase activity by diethyldithiocarbamate which is a specific inhibitor of CYP2E1 [8]. The extent of inhibition was comparable (90−100%) in liver microsomes from newborns and adults, with doses of 100, 250 and 500 μM diethyldithiocarbamate. This suggested a similar affinity of the inhibitor for CYP2E1 whatever the age. It also confirmed that CYP2E1 was the major isoform involved in the 6-hydroxylation of chlorzoxazone.

CYP2E1 RNA ontogenesis. The accumulation of *CYP2E1* transcript in the developing liver was assessed by slot-blot analysis in 70 liver samples previously used in protein studies. We used two probes located in different regions of the cDNA to overcome a possible degradation of RNA: probe B2 hybridized to exon 1 and probe C corresponded to exon 5. Results were expressed as the ratio between the densitometric values of CYP2E1 RNA and 28S rRNA: values obtained with probe C were highly correlated with those obtained with probe B2 (r = 0.98; data not shown).

The densitometric quantitation of CYP2E1 RNA indicated a moderate increase of the signal intensity throughout the early postnatal development (Fig. 3) in contrast with the abrupt rise of the protein content during the first days following birth (Fig. 1). This suggests the involvement of a post-translational event during the early neonatal period. Actually, the average concentration of CYP2E1 RNA was 15-fold higher in adults than during the early neonatal period. The major increase of the transcript level occurred in newborns aged 1−3 months and reached 50% of the adult value in infants aged 3−12 months. Due to the size of samples, no RNA measurement was performed in children aged more than 1 year. After one month of age, variations of the transcript level roughly paralleled the increase of the microsomal CYP2E1 content: this correlation is consistent with the proposal that developmental regulation of the *CYP2E1* gene occurs at the transcriptional level during this period.

Methylation analysis of genomic DNA. This study was undertaken to determine the cytosine methylation profile of *Hpa*II/*Msp*I sites located within the 5′ flanking region (≈1 kbp), the first exon and the first intron of the *CYP2E1* gene. *Msp*I cuts at the sequence CCGG, regardless of the methylation status of the internal cytosine, whereas digestion by *Hpa*II is prevented by methylation. We examined genomic DNA isolated from fetuses believed to be negative controls, from adults as full positives, and from newborns selected for their different levels of CYP2E1 RNA. DNA samples were digested to completion with *Hpa*II or *Msp*I and analyzed by Southern blot with probes allowing adjacent regions to be explored.

In a first attempt, we investigated the 5′ flanking region of the *CYP2E1* gene. Southern blots were hybridized with probe A covering the region around the transcription start site from position −1013 to 72. In adult DNA digested with *Hpa*II, and in both fetal and adult DNA cut with *Msp*I, we observed a 430-bp band adjacent to the first exon (H2-H3). In contrast, this band was absent from fetal samples digested with *Hpa*II (data not shown). This preliminary result indicated a role of hypermethylation in the transcriptional control of the *CYP2E1* gene and suggested that either H2 or H3 sites were methylated in the human fetus.

Next, we examined the methylation pattern of the promoter region, the first exon and the first intron of the *CYP2E1* gene with a 1.2-kbp probe B1 derived from the 1.7-kbp fragment B cleaved with *Xba*I (Fig. 4). This probe covered the region from −197 to 1022, and allowed us to explore 10 *Hpa*II/*Msp*I sites generating fragments spanning 430, 285, 200, 190, 180 and <50 bp. The expected digestion products were obtained in all samples cut with *Msp*I (Fig. 5A). We confirmed that the 430-bp band adjacent to the first exon was missing in *Hpa*II digests of fetal samples, and we observed two additional fragments of 630 bp and 810 bp. These fragments were present only in fetal samples and were detected in two fetuses aged 21−23 weeks. We interpreted this result as a methylation of both sites H3 and H4 (generating the 810-bp fragment) or site H3 only (fragments of 630 bp and 180 bp) in fetal samples. Demethylation at site H3 yielded the 430-bp band in the *Hpa*II digest, observed as a weak signal in the newborn expressing low levels of CYP2E1 RNA (NB1); the intensity of the 430-bp band increased in newborns with a high level of transcript (NB2) and in the adult.

In the 280−310-bp region, bands corresponded to fragments H7/9-H10 (285, 310 and 318 bp) as shown in *Msp*I and *Hpa*II digests. Additionally, probe B1 hybridized with 380−400 bp fragments in fetal liver DNA digested with *Hpa*II. The intensity of this hybridization signal at 380−400 bp was markedly reduced in DNA from newborns expressing the *CYP2E1* gene and was undetectable in adult samples. This suggested that the 380−400-bp fragments resulted from the demethylation at sites H5-H6 and H10-H11, which are separated by 380−400 bp, while the internal sites H7, H8 and H9 were methylated. These sites became progressively demethylated in *CYP2E1*-expressing livers, leading to the complete loss of the 380−400-bp bands in the adult preparation. The intensity of the signal at 380−400 bp was weaker than those of the 280−310-bp bands and indicated that sites H7, H8 and H9 were predominantly but not fully demethylated in these fetal livers. Since the *CYP2E1* gene is unique, this reflects the balance existing between liver cells with sites H7−9 methylated and cells with sites H7−9 demethylated. Their progressive demethylation explained the intermediate length of DNA fragments ranging over 280−310 bp. The bands

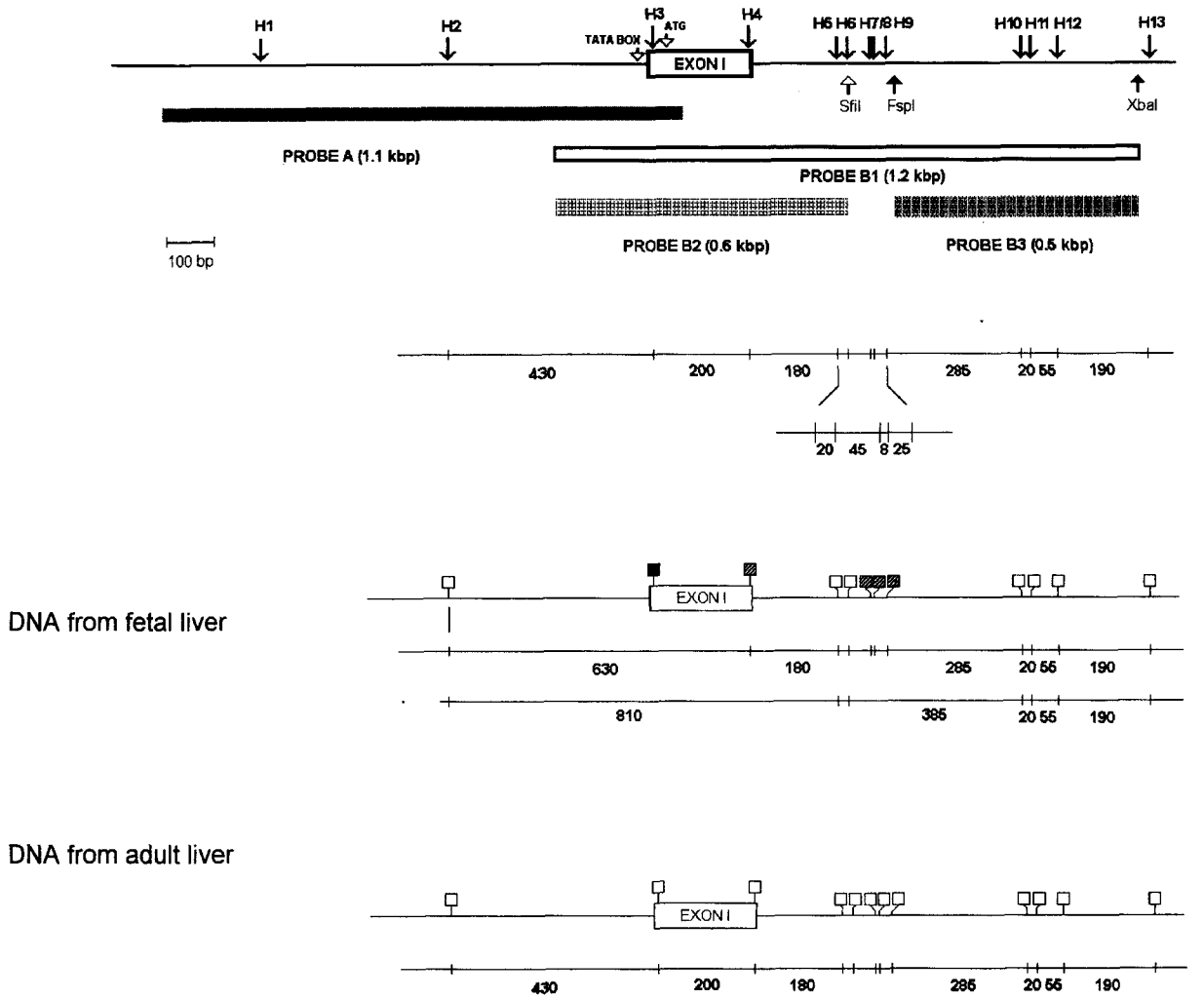

Fig. 4. Map of the 5′ flanking region, first exon and first intron of the human *CYP2E1* gene and schematic representation of restriction fragments generated by digestion of fetal and adult DNA by *Hpa*II/*Msp*I. Open box indicates the position of the first exon. Vertical arrowheads denote the restriction sites for *Hpa*II and *Msp*I, numbered from H1 to H13. The fragments used for hybridization are shown below: probe A is a 1.1-kbp fragment obtained by PCR; probe B1 is a 1.2-kbp *Xba*I fragment derived from the 1.7-kbp PCR product B; probes B2 and B3, ≈0.6 and ≈0.5 kbp, respectively obtained by *Sfi*I and *Fsp*I cleavage of fragment B1. The lower part summarizes the schematic representation of the methylation pattern of the *CYP2E1* gene in fetal and adult livers. The methylation status of individual sites and the length of the generated fragments are indicated: methylated sites are represented by closed squares, the demethylated sites by open squares and the partially methylated sites by hatched squares.

at 180−200 bp represented fragments H4-H5, H4−H6 and H12-H13 all located in the first intron.

Finally, a third set of hybridizations was performed with probes B2 and B3 to avoid possible ambiguities in the interpretation of the restriction map. The 600-bp probe B2 (positions −197 to 416) and the 500-bp probe B3 (positions 512−1022) were obtained from B1 by *Sfi*I and *Fsp*I digestion of the 1.2-kbp fragment (Fig. 4). The probe B2 hybridized only with the 430-bp fragment (H2-H3) and with fragments spanning 180−200 bp (H3-H4, H4-H5 and H4−H6) as shown in *Msp*I digests (Fig. 5B). In fetuses, the absence of the 430-bp fragment in *Hpa*II digests clearly indicated hypermethylation at site H3, leading to the presence of a band at 630 bp and a band at 810 bp probably resulting from a partial methylation at site H4. The presence of additional bands with higher molecular masses also suggested at least partial methylation of several other sites.

Probe B3 allowed us to explore the region between H9 and H13. The 180/200-bp and the 280/310-bp fragments resulted from cleavage at sites H9, H10-11, H12 and H13, as shown with *Msp*I digests. When hybridization was performed with the probe B3 (Fig. 5C), the presence of a 380-bp fragment was noticed in fetal samples, was barely detectable in newborns and was totally absent from adult samples digested with *Hpa*II. The absence of bands with higher molecular masses confirmed the previous assumption that sites H5-H6 and H10−H12 were demethylated. Conversely, this result indicated that in the 380−400-bp fragments, sites H7, H8 and H9 are methylated. As noticed with probe B1, the intensity of the 380−400-bp fragments was lower than those of 280−310-bp fragments. This confirmed the previous assumption that sites H7, H8 and H9 were partially methylated in the fetal liver, generating either the 280−310-bp fragments (H7, H8, H9 demethylated) or the 380−400-bp fragments (H7, H8, H9 methylated).

DISCUSSION

Data collected from ontogenic studies have demonstrated that the *P*-450 system develops early during the fetal life [38, 39], but displays several differences with the adult system:

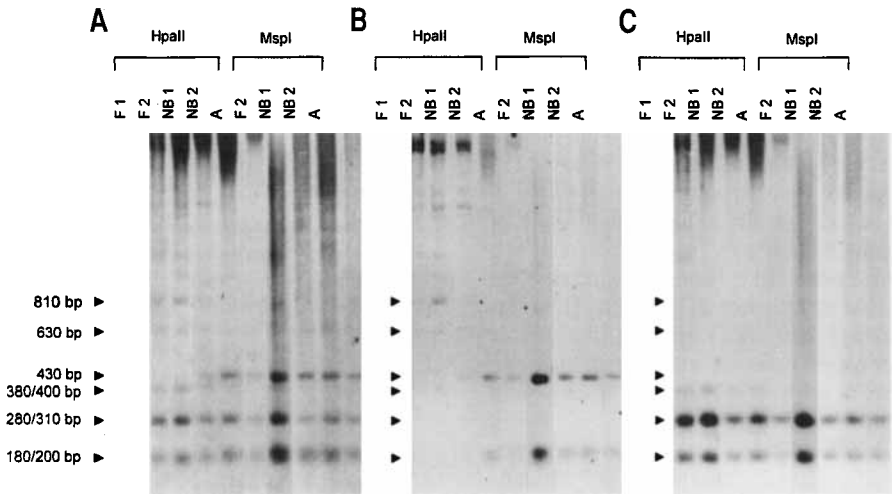

Fig. 5. Southern blot analysis of genomic DNA from human liver. The methylation pattern of the promoter region, the first exon and the first intron of the *CYP2E1* gene was assessed by Southern blot analysis: 30 µg genomic DNA digested by the methylation-sensitive isoschizomers *Hpa*II and *Msp*I were electrophoresed on 1.8% agarose gels, transferred to Hybond N filters and hybridized with probes shown in Fig. 4. F1, fetus aged 21 weeks; F2, fetus aged 23 weeks; NB1, newborn expressing a low level of CYP2E1 RNA; NB2, newborn expressing a high level of CYP2E1 RNA; A, adult. The approximate size of the analyzed fragments is marked on the left side. (A) Hybridization with probe B1. This probe spans 1.2 kbp and covers the promoter region, the first exon and the first intron: 10 *Hpa*II/*Msp*I sites were located in this region and generated fragments of 430, 285, 200, 180 and < 50 bp. (B) Hybridization with probe B2. It hybridized with fragments H2-H3, H3-H4 and H4-H5 of 430 bp and 180–200 bp. (C) Hybridization with probe B3. Fragments of 285 bp (H9-H10) and 190 bp (H12-H13) hybridized with this probe.

CYP3A7 is a fetal-specific form of *P*-450, whereas CYP3A4 and CYP2C proteins were totally absent from the fetal liver [39, 40] even if low amounts of RNA could be detected by PCR [41]. Similarly, the polymorphic CYP2D6 was not present in fetal liver but mostly developed after birth [29].

In the present study, the expression and regulation of *CYP2E1* have been investigated in human livers during development. The expression of immunochemically detected CYP2E1 protein and the level of its associated chlorzoxazone 6-hydroxylase activity did not follow the pattern of expression of total *P*450. Thus, no CYP2E1 protein was detectable in fetal microsomes but its content surged immediately after birth, whatever the gestational age. This strongly suggested that an event directly related to parturition was implicated in the regulatory process rather than temporal maturation by itself. The lack of expression in all fetal samples indicated that conditions of abortion (spontaneous or induced) and the various pathologies which caused spontaneous abortions had no effect on the physiological process of CYP2E1 gene activation.

The surge of CYP2E1 within the first hours after birth might be related to a physiological event: of interest, CYP2E1 could play a key role during the early neonatal metabolism [42]. The onset of CYP2E1 was concomitant with important nutritional changes in the newborn diet, relative to modifications in the frequency of feedings (with partial fasting) and with composition of the diet: it changed from a glucose-rich diet continuously supplied to the fetus through the placenta to a discontinuous lipid-rich diet provided by breast feeding or by formulated milk. CYP2E1 may be involved in two gluconeogenic pathways from acetone, as proposed by Cassaza et al. [12]. The initial step is the hydroxylation of acetone to acetol, which represented the branch point between these two pathways: one leads to the formation of methylglyoxal and the second converts acetol into 1,2-propanediol [13]. The increase in acetone concentration and the presence of acetol and 1,2-propanediol have been reported in the plasma of fasting humans [43]. In rats given acetone, a significant decrease in degradation of CYP2E protein caused an apparent increase of its concentration in microsomal membranes without alteration of the RNA accumulation [19]. This mechanism could explain the rapid surge of CYP2E1 protein during the first hours following parturition in humans: the neonate is in a fasting situation immediately after birth and increased its concentration of plasma ketone bodies during the first hours following birth [44]. These newborns have a degree of ketosis and a turnover of ketone bodies that are achieved by the adult only after two days of total starvation [45]. Ketone bodies could then stabilize the low amount of existing CYP2E1 protein in the liver without stimulation of the transcription rate or accumulation of its transcript.

After 1 month of age, the rise in the CYP2E1 protein content followed the RNA accumulation, and could resulted from either a stabilization of RNA or an activation of the transcriptional activity during this period. The influence of the methylation state of *CYP2E1* gene upon its expression was clearly demonstrated in rat liver: CYP2E RNA accumulated after birth resulting from the transcriptional activation of the gene [21] and was accompanied by the demethylation of some cytosine residues in the region upstream from the transcription start site [22]. DNA methylation appears to play an important role in the general control of gene expression [46, 47]. A general feature is the negative correlation between the degree of DNA methylation and the transcription rate of certain genes, particularly in the dinucleotide pair CpG at the 5′ end of genes. Although the exact mechanism underlying the control of transcription is not completely understood, DNA methylation may prevent or reduce the binding of *trans*-acting factors to *cis*-acting elements [47]. Recently, Yokomori et al. [48] reported that methylation of the sex-specific promoter of the mouse *Cyp2d9* gene totally prevented the binding of the heteromeric transcription factor GABP and abolished the expression of the *Cyp2d9* gene in male mice.

Attempts have been conducted in human livers to correlate the transcriptional activity with the hypermethylation of the *CYP2E1* gene. Jones et al. [49] using cDNA-derived probes reported a partial methylation of the 5′ part of the gene in both adult and fetal DNA and noted the presence of two small *Hpa*II fragments (0.7 and 0.55 kbp long) in fetuses aged 12–17 weeks, but not in adult livers. Unfortunately, the location of methylated sites was not defined. In another study, a 3′ probe corresponding

to positions 749–1623 of the cDNA was used to characterize the methylation of genomic DNA isolated from different tissues: the authors concluded that the pattern of methylation was identical within the same tissue of several individuals, but differed from one tissue to another [50]. Thus, lung DNA was less methylated than liver DNA, although the relative RNA level was much higher in the liver: in that case, hypomethylation of the 3′ end of the coding region resulted in the reduced expression of the gene. Similar conclusions were drawn from the comparison of normal and tumoral lung tissues [51]. This is in contradiction with data collected from the expression of aldolase B in the liver: the heavily methylated 3′ half and 3′ flanking region had no influence on the level of expression, in contrast with the close association observed between expression and the methylation status of the 5′ flanking and 5′ half region [52].

Herein, the onset of CYP2E1 RNA accumulation was tentatively correlated with specific demethylations of CpG residues precisely located within the 5′ flanking region (≈1 kbp upstream from the transcription start site), the first exon and the first intron. First, the fragments generated by digestion to completion with *Msp*I exhibited the expected size in the region covering 1 kbp upstream from the transcription start site, exon 1 and intron 1. Second, the digestion pattern was identical with *Hpa*II and *Msp*I in adult liver samples and suggested that no hypermethylation occurred in this region. Lastly, different restriction profiles have been obtained when genomic DNA was extracted from fetal, newborn and adult livers, indicating that the transcriptional activation of human *CYP2E1* gene involved the demethylation of CpG residues within the first exon and the first intron of the gene. We clearly demonstrated that the hypomethylation of CpG sites was associated with the accumulation of CYP2E1 RNA. In this respect, the location of methylated CpG residues could play a major role in the modulation of the transcription rate. Of interest, the site H3 located 34 bp downstream the TATA box is methylated in samples with no or little CYP2E1 RNA: according to the current hypothesis, methylation might prevent the binding of proteins involved in the transcription complex. Interestingly, specific methylation sites have been localized in the same DNA region as DNase-I-hypersensitive sites of promoters of albumin and α-fetoprotein genes and are believed to control the transcription process [53]. The mapping of DNase-I-hypersensitive sites in the 5′ region of *CYP2E1* gene in fetal and adult liver is currently under investigation to define conclusively the involvement of this mechanism in the regulation of transcriptional activity during the neonatal period.

In conclusion, two mechanisms seem to be associated in the regulation process of the human hepatic *CYP2E1* during the neonatal period: a stabilization of the CYP2E1 protein caused by the high ketosis occurring during the first days of life; a demethylation at the 5′ end of the gene leading to an accumulation of RNA after 1 month of age. To date, no explanation can account for the physiological change in the methylation status of the *CYP2E1* gene promoter during this period.

Authors are indebted to Drs F. Narcy (*Dept Anatomie Pathologique*), and P. Mussat (*Dept Neonatologie, hôpital Cochin*, Paris), and to Dr G. Cheron (*Urgence Pédiatrique, hôpital Necker*, Paris) for providing fetal and neonatal human liver samples. Isabel Vieira is supported by a PhD fellowship (BD/2085/92-ID) from the *Junta Nacional de Investigaçao Cientifica e Tecnologica* (Portugal).

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